Polyhydroxybutyrate and glycogen production in photobioreactors inoculated with wastewater borne cyanobacteria monocultures

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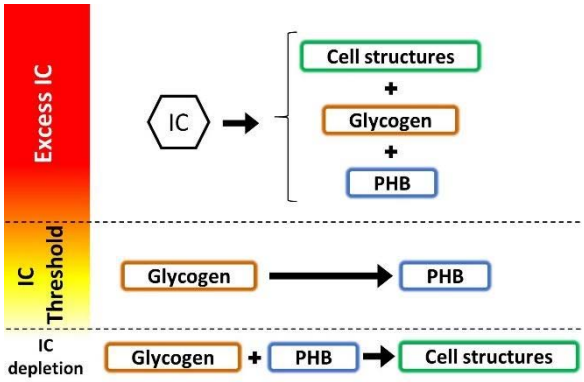
Graphical abstract



Abstract

The aim of this study was to investigate the PHB and glycogen accumulation dynamics in two photobioreactors inoculated with different monocultures of wastewater-borne cyanobacteria, using a three-stage feeding strategy (growth phase, feast-famine phase and feast phase). Two cyanobacterial monocultures containing members of *Synechocystis* sp. or *Synechococcus* sp. were collected from treated wastewater and inoculated in lab-scale photobioreactors to evaluate the PHB and glycogen accumulation. A third photobioreactor with a complex microbial community grown in real wastewater was also

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set up. During each experimental phase different concentrations of inorganic carbon were applied to the cultures, these shifts allowed to discern the accumulation mechanism of carbon storage polymers (PHB and glycogen) in cyanobacteria. Conversion of one into the other was directly related to the carbon content. The highest PHB and glycogen contents (5.04%$_{dcw}$ and 69%$_{dcw}$, respectively) were achieved for *Synechocystis* sp.

Keywords

PHA · biopolymers · wastewater-borne cyanobacteria · feast-famine · Inorganic carbon

Highlights

- *Synechocystis* sp. was the most PHB productive species.
- PHB and glycogen content are strongly correlated with inorganic carbon availability.
- There is a carbon threshold that triggers the transformation of glycogen to PHB.
- High concentration of carbon boosted both PHB and mostly glycogen accumulation.

1. Introduction

In the last 50 years, global plastic production has increased twenty times (up to 322 million tonnes in 2015) and is expected to continue growing, doubling the current production by 2035 (European Comission, 2015). Approximately, 60% of plastic produced worldwide (4900 million tonnes) has already been discarded to landfills or natural ecosystems. Taking into consideration that these plastics are barely decomposable, environmental issues and serious pollution problems are arising in basically all of the environmental compartments and trophic levels. The need to find sustainable alternatives, is therefore critical.

Polyhydroxybutyrate (PHB) has gained interest as a solid alternative to conventional plastics, as, it can be degraded and mineralized. Additionally, it has similar characteristics to traditional plastics from the petrochemical industry (such as polypropylene), including an equal response to extrusion, injection moulding, or fibre spinning (Drosg et al., 2015; Singh et al., 2017; Troschl et al., 2017). Since its first commercialization in 2004 by the company Metabolix™, PHB market has progressively grown and many other manufacturers have arisen. However, PHB price is still far from being competitive (Singh et al., 2017; Vaidya et al., 2019).

The current established biotechnological process uses heterotrophic biomass to produce PHB, as cultures can be easily scaled up and, as of now, they have shown the highest PHB production rates (Drosg et al., 2015; Singh and Mallick, 2017; Troschl et al., 2017). However, heterotrophic bacteria usually require large amounts of organic carbon from vegetal origin, which lead to competition for arable land (Calixto et al., 2018; Lau et al., 2015; Troschl et al., 2017). The use of organic carbon sources and oxygenation requirements for the aerobic metabolism of these microorganisms, account for approximately 30-50% of the PHB biosynthesis costs (Costa et al., 2018; Singh and Mallick, 2017; Troschl et al., 2017). Taking this into consideration, cyanobacteria have become a promising alternative to produce PHB, as they can overcome all the drawbacks aforementioned. They can produce bioplastics autotrophically, using sunlight and recycling CO_2 from the atmosphere or industrial effluents. They also have low nutritional requirements, so the production costs in terms of growth media, could decrease substantially. Furthermore, ethical conflicts of using arable areas could be avoided, as cyanobacteria can be cultivated in many non-agricultural sites such as roofs or deserted regions (Costa et al., 2018; Drosg et al., 2015; Lau et al., 2015; Singh and Mallick, 2017). Nevertheless, PHB production by cyanobacteria still needs to overcome other challenges,

especially low production rates achieved so far in comparison to those obtained by chemosynthesis (heterotrophic bacteria) (Drosg et al., 2015; Lau et al., 2015; Singh and Mallick, 2017). It is therefore essential to optimize the autotrophic PHB production, in order to increase the intracellular concentration of PHB, and minimize the production costs to become a competitive production alternative.

Within this context, a good alternative to produce cyanobacterial biomass in a cost-effective and eco-friendly manner is to use wastewater effluents as a nutrient source (Acién et al., 2012). Acién et al., (2012) concluded that using wastewater could reduce the production costs from 3€/kg to 1.8€/kg

Concerning the enhancement of PHB concentration, one of the most studied approaches is to grow cyanobacteria in a nitrogen (N) and phosphorus (P) starved media (Arias et al., 2018a, 2018b; Singh et al., 2017; Singh and Mallick, 2017). Recently, Arias et al., (2018a) also studied a strategy to enhance the carbon uptake efficiency by adding carbon in a feast-famine regime, which consists of repeated cycles of full availability of carbon (feast phase), followed by its complete absence (famine phase). This feast-famine regime has been widely applied in the production of PHB in mixed heterotrophic bacterial cultures, as it causes a positive selective pressure over the PHB storing microbial populations (Arias et al., 2018a; Koller et al., 2008; Wijeyekoon et al., 2018). Furthermore, it also generates an unbalanced growth that stimulates polymer accumulation (PHB and glycogen) when carbon is fully available during the subsequent feast phase (Reis et al., 2003).

On one hand, the biosynthesis of PHB competes with glycogen synthesis for the 3-phosphoglycerate (3-PG) (metabolic intermediate generated during carbon assimilation in the Calvin cycle). Thus, great part of the fixed carbon is used for glycogen synthesis instead of PHB production (Singh et al., 2017). On the other hand, glycogen can be

oxidized to produce energy and reduce the power required for PHB synthesis (Kamravamanesh et al., 2019). Meaning that in order to understand the effect of culturing conditions on the biosynthesis of carbon storage biopolymers, both glycogen and PHB accumulation must be studied together.

Previous studies on accumulation of PHB and glycogen used cyanobacteria obtained from culture collections. These strains are probably unadapted to wastewater growing conditions and they will rarely grow in this media, hindering the use of wastewater as nutrient feedstock. The present study evaluates, for the first time, PHB and glycogen accumulation mechanisms of cyanobacterial monocultures selected from photobioreactors (PBR) fed with wastewater. The selected cyanobacteria are highly adapted to these conditions, and thus, the most promising to be applied in industrial applications using wastewater as a nutrient source. The main objective of this work was to select the most effective producing PHB species, following a three-phase cultivation strategy (growth, feast and famine, and feast), and obtain an insight on the PHB and glycogen accumulation mechanisms of these species under these changing conditions.

2. Materials and methods

2.1. Media and reagents

Cyanobacterial cultures were isolated, cultured and maintained in BG-11 medium, that consisted of: 1500 $mg \cdot L^{-1}$ $NaNO_3$, 31.4 $mg \cdot L^{-1}$ K_2HPO_4, 36 $mg \cdot L^{-1}$ $MgSO_4$, 36.7 $mg \cdot L^{-1}$ $CaCl_2 \cdot 2H_2O$, 20 $mg \cdot L^{-1}$ Na_2CO_3, 1 $mg \cdot L^{-1}$ NaMgEDTA, 5.6 $mg \cdot L^{-1}$ citric acid, 6 $mg \cdot L^{-1}$ Ferric ammonium citrate and 120 $mg \cdot L^{-1}$ $NaHCO_3$. To prepare solid medium BG-11 was supplemented with 1% bacteriological agar. The BG-11 medium and the bacteriological agar were autoclaved separately (at double strength) and mixed when cooled at 50 °C, to avoid formation of toxic decomposition products (Allen, 1968).

The reagents for the modified BG-11 used in the reactors (K_2HPO_4, $NaNO_3$, $NaHCO_3$, $CaCl_2 \cdot 2H_2O$, NaOH, Na_2EDTA, $NaHCO_3$) and the reagents needed for NO_3^-, NO_2^-, NH_3 and P analysis were obtained from Panreac (Barcelona, Spain). $MgSO_4 \cdot 7H_2O$, $C_6H_8FeNO_7$ (ammonium ferric citrate), $C_6H_8O_7$ (citric acid), HCl, chloroform ($CHCl_3$) and *D*-glucose were purchased from Scharlau (Barcelona, Spain). Prepared BG-11 broth, CH_3OH, H_2SO_4, $C_{17}H_{36}$ (heptadecane) and PHB-PHV (86:14% wt, CAS 80181-31-3) copolymer standard were purchased from Sigma-Aldrich (St. Louis, US).

2.2.Procurement of a wastewater borne cyanobacterial monoculture

Samples for cyanobacteria isolation were taken from two different photobioreactors, in which a complex microbial community containing cyanobacteria and microalgae were cultivated using wastewater as a feedstock. One of the reactors was a full-scale hybrid tubular horizontal photobioreactor (HTH-PBR) located at the experimental campus Agròpolis of Universitat Politècnica de Catalunya (UPC), Viladecans, Barcelona (41.288 N, and 2.043 E UTM). This HTH-PBR had a total useful volume of 11.7 m^3 and was daily fed with 2.3 m^3 of a mix of water from a drainage channel (agricultural run-off from fields in the area) and domestic wastewater from an aerated septic tank, with a ratio 6:1. The composition of the influent wastewater was as follows: 1.04 mg N-$NH_4^+ \cdot L^{-1}$, 3.76 mg N-$NO_3^- \cdot L^{-1}$; 0.33 mg N- $NO_2^- \cdot L^{-1}$; 0.42 mgP$\cdot L^{-1}$ total soluble phosphorus, 18.6 mgC$\cdot L^{-1}$ total organic carbon (TOC), 49.4 mgC$\cdot L^{-1}$ total inorganic carbon (TIC) and 62.6 mgTSS$\cdot L^{-1}$ total suspended solids (TSS). More information about the operation and design of this HTH-PBR can be found elsewhere (García-Galán et al., 2018; García et al., 2018). The second reactor was a lab-scale 30 L closed photobioreactor (30L-PBR) fed daily on semi-continuous mode with 3 L of digestate diluted in secondary effluent from a high-rate algal pond (fed with urban wastewater) in a ratio 1:50 as described previously by Arias et al., (2017). Both photobioreactors were chosen assuming that the cyanobacteria species

isolated from them could reliably grow and compete in the wastewater cultures. To facilitate sample manipulation and cyanobacteria recovery, samples were homogenized to disaggregate flocs prior to isolation. Two different methods were tested: 1) Sonication of biomass samples in an ultrasonic bath at 360 W during 45 s as described by Abzazou et al., (2015). 2) Homogenization of samples with a Polytron™ PT 2500E (Polytron, Montreal, Canada) at 10000 rpm per 1 min.

Homogenized samples were inoculated in BG-11 plates by direct streaking or after serial dilution on saline media. Agar plates were incubated until isolated colonies corresponding to cyanobacteria were observed (approximately 2 weeks). To obtain cyanobacterial monocultures a single isolated colony was transferred to a new plate. In case more than one type of cyanobacteria would have formed in the same plate, each cyanobacteria colony was re-plated separately. All plates were incubated at 30 ± 2 °C under 15 hours light: 9 hour dark cycles and were illuminated with a 14W cool-white LED lights.

2.3. Identification of cyanobacteria

The cultured cyanobacteria were observed by both, a bright light microscope (Motic, China) equipped with a camera (Fi2, Nikon, Japan) and a fluorescence microscope (Eclipse E200, Nikon, Japan) using the NIS-Element viewer® software. Cyanobacteria were identified and classified following the morphological descriptions provided by the cyanobacteria database CyanoDB (Komárek J. & Hauer T, 2013) and a reference taxonomic book (Streble and Krauter, 2018).

Considering the likely presence of accompanying heterotrophic communities, a molecular characterization of cyanobacterial monocultures and photobioreactor biomass was achieved by clone library analysis based on 16S rRNA gene amplification. For the initial characterization of cyanobacterial monocultures, several colonies grown on BG-11 plates were suspended in 50 µl of PCR quality water and total DNA was extracted using the

PowerSoil DNA extraction kit (MoBio, USA) following manufacturer instructions. For bioreactor biomass, 10 ml of reactor media were centrifuged at 4500 rpm for 20 min, and the pelleted biomass was subjected to DNA extraction using the same kit. Bacterial 16S rRNA gene fragments were amplified using primers 27F and 1492R (Weisburg et al., 1991). The PCR mixture was prepared in the PureTaqTMReady-To-GoTM PCR beads tubes (GE healthcare, United Kingdom) in a final volume of 25 µl containing 25 pmol of each primer (Sigma-Aldrich, Steinheim, Germany). PCR amplifications were performed on an Eppendorf Mastercycler as described previously (Gallego et al., 2014). Amplified 16S rRNA gene fragments were cloned using the pGEM-T Easy Vector System (Promega, Madison, WI, USA). To analyze transformants, plasmids were purified using the GeneJet Plasmid Miniprep Kit (Thermo Scientific), and cloned fragments were amplified by PCR using primers GC40-63F and 518R for further DGGE analysis as described previously (Gallego et al., 2014).

Clones with identical DGGE patterns were grouped, and representative clones corresponding to each DGGE pattern were sequenced using primers 27F and 1492R at Macrogen Europe. DNA sequencing runs were assembled to obtain almost full length 16S rRNA gene sequences, aligned and manually adjusted using the BioEdit Software (Hall, 1999) Aligned sequences were clustered into operational taxonomic units (OTUs) using the DNA distance tool implemented in BioEdit with a sequence identity cut off at 99%, and analyzed for the presence of chimeras using the DECIPHER's Find Chimeras web tool (Wright et al., 2012). Representative sequences from each OTU were compared to the non-redundant database of sequences deposited at the National Center for Biotechnology (NCBI; http://www.ncbi.nlm.nih.org/blast/) using BLASTN algorithm, and classified using the classifier tool of the Ribosomal Database Project (RDP;

http://rdp.cme.msu.edu/). The obtained 16S rRNA gene sequences were deposited in the GenBank database with access numbers MN493561 to MN493580.

2.4. Upscaling: from plate to a laboratory scale photobioreactor

To scale up cyanobacterial monocultures, single colonies recovered from BG-11 plates were transferred to 2 mL of liquid BG-11 medium contained in a 15 mL test tube. When the colour of the culture changed to a blue-green colour (400-500 NTU), biomass was scaled up to a larger volume using a small scale-up ratio of 1:5 to achieve proper cyanobacterial growth. This ratio ensures a proper culture growth, while higher ratios sometimes lead to culture failure (Andersen, 2005).

This process was repeated sequentially from 2 mL to 1000 mL. Eventually, those 1000 mL were the inoculum for the 2,5 L lab scale photobioreactors. Test tubes were used for volumes from 2 to 10 mL. These cultures were kept at 30 ± 2 ºC under 1 klx illumination. Erlenmeyer flasks were used for volumes from 50 mL to 1000 mL. These cultures were continuously agitated either by means of sterile air bubbling or by magnetic agitation. The air flow rate or the agitation velocity were set at a speed high enough to avoid settling of the biomass. Cultures were kept under 2.1 klx illumination at 30 ± 2 ºC. From 250 mL onwards, aeration with compressed air was needed. It took between 7-15 days to scale up from one volume to another.

2.5.Lab scale photobioreactors set up

PHB and glycogen accumulation experiments were carried out in 2.5 L lab- scale photobioreactors. These were closed polymethacrylate cylinders with a diameter of 11 cm and a total volume of 3 L. Each reactor was inoculated with 0.5 L of the studied cyanobacterial culture and 2 L of BG-11 media. In the case of the mixed culture, 2.5 L obtained directly from the HTH-PBR were used. Reactors were continuously agitated with a magnetic stirrer (VELP scientifica, Usmate, Italy) ensuring a complete mixing.

Reactors were submitted to 15:9 h light:dark phases and were illuminated by two external 14 W cool-white LED lights providing a medium illuminance of 2.1 klx. Illuminance at different reactor points was measured with a luxmeter (HI 97500, HANNA instruments, Italy). Temperature was regularly measured and maintained at 30 (±2) °C.

Three different phases were applied to each reactor in order to enhance the PHB and glycogen accumulation (Figure 1):

1. *Growth phase*: for the reactors inoculated with cyanobacterial monocultures, N, P and C were added at a concentration that cells could exhaust when biomass would have reached approximately a concentration of 1 $g \cdot L^{-1}$ VSS. For the mixed culture reactor, 2.5 L obtained from the HTH-PBR were used, so only the nutrients from the HTH-PBR sample were present. N, P and C were regularly measured. The duration of this phase depended on the nutrient uptake rate of each species (from 20 to 30 days). CO_2 injection was used to control pH during this phase, until N and P were almost depleted. In the mixed PBR, due to its high particulate inorganic carbon concentration, HCl was used.
2. *Feast-Famine phase*: the aim of this phase was to improve C uptake efficiency. It lasted 1 week and started when N, P and C from the previous phase were depleted. During this phase, C was only available for 6h per day (feast) and non-available for the rest of the day (famine). In the case of the experiment with *Synechocystis* sp, C was added as CO_2 during the first 3 h by a pH controller. Due to the difficulties observed in controlling the amount of C added by CO_2 injection, bicarbonate was used for the feast and famine phase in the experiments with *Synechococcus* sp. and mixed culture. In these cases, 4 mL of bicarbonate with a concentration of 2 g $C \cdot L^{-1}$ were added to reach 5 mg $C \cdot L^{-1}$ at the beginning of each feast and famine cycle. This amount of bicarbonate was expected to be consumed

after 6 h from the start of the famine phase. Regarding pH control, in order to achieve C depletion during the famine phase, it was performed adding HCl 0.1 M (except in *Synechocystis* sp. reactor, in which CO_2 was used).

3. *Feast phase*: 120 mg $C \cdot L^{-1}$ as bicarbonate were added to each culture at the beginning of the feast phase, whereas CO_2 was also injected to control the pH in all the reactors. The availability of C was expected to boost the PHB and glycogen accumulation.

The pH was continuously measured by a pH probe (HI1001, HANNA instruments, Italy) and kept at an optimum level (between 7.5-9) by means of an automatic pH controller (HI 8711, HANNA instruments, Italy) that activated CO_2 or HCl (0.1 M) injection, depending on the current culture phase. Bicarbonate additions where done using a peristaltic pump that was activated by a timer during 1 min each day at the beginning of the feast-famine cycle. (see Figure 1).

Due to different reasons, CO_2 has been considered as the best carbon source during growth and feast phase (Markou et al., 2014). During feast and famine phase, CO_2 was also used as a carbon source in *Synechocystis* sp. reactor. However, the amount of carbon added by CO_2 injections was difficult to control, as it depends on many factors (temperature, reactor agitation, gas flow, type of diffuser, liquid column…). Therefore, bicarbonate was used instead during feast and famine phase in the subsequent *Synechococcus* sp. and mixed reactors. This better control allows to add the exact amount of IC that the culture will consume in 6h of feast cycle, leading to a complete IC exhaustion during the famine period.

2.6.Analytical methods

2.6.1. Nutrients analysis

Cultures were periodically analysed to evaluate nutrient uptake and cyanobacteria growth rate. Dissolved and total inorganic carbon (DIC and TIC), as well as total dissolved carbon (TDC) and total carbon (TC) were measured at the beginning of the experiment and at the end of each experimental phase (growth phase, feast-famine phase and feast phase). DIC or TIC were measured once a week. Other C forms were calculated as follows:

- Total organic carbon (TOC) = TC-TIC
- Dissolved organic carbon (DOC) = TDC - DIC
- Particulate organic carbon (POC) = TOC-DOC
- Particulate inorganic carbon (PIC) = TIC-DIC

Total nitrogen (TN), total dissolved nitrogen (TDN), nitrite ($N\text{-}NO_2$), nitrate ($N\text{-}NO_3$) and ammonia ($N\text{-}NH_3$) were measured with a similar pattern. Dissolved organic nitrogen (DON) and particulate nitrogen (PN) were calculated as follows:

- Dissolved organic nitrogen (DON) = TDN - $N\text{-}NO_2$ - $N\text{-}NO_3$ - $N\text{-}NH_3$
- Particulate nitrogen (PN) = TN – DON - $N\text{-}NO_2$ - $N\text{-}NO_3$ - $N\text{-}NH_3$

Nitrate, which is the most important form of inorganic nitrogen in the culture medium, was measured twice per week during the growing phase to monitor N uptake.

Total phosphorus (TP), total dissolved phosphorus (TDP) and orthophosphate ($P\text{-}PO_4$) were also measured at the beginning of the experiment and at the end of each phase. Orthophosphates were measured twice per week to monitor P uptake. Dissolved organic phosphorus and particulate phosphorus were calculated as follows:

- Dissolved organic phosphorus (DOP) = TDP- $P\text{-}PO_4$
- Particulate phosphorus (PP) = TP– DOP - $P\text{-}PO_4$

TC, TDC, TIC, DIC, and TN concentrations were analysed using a C/N analyser (2005, Analytikjena, Germany). N-NO_2 and N-NO_3 were measured by the colorimetric methods described in Standard Methods (methodologies 4500-NO_2^- and 4500-NO_3^-, respectively) (Clesceri et al., 1999). P-PO_4, TP and TDP were analysed following the colorimetric methodologies 4500-PE and 4500-P described in Standard Methods (Clesceri et al., 1999). All dissolved forms were previously filtered through 1-3 µm pore glass microfiber filter.

Total suspended solids (TSS) and volatile suspended solids (VSS) were measured 2 days per week following gravimetric methods 2540C and 2540 D described in Standard Methods (Clesceri et al., 1999).

2.6.2. PHB and glycogen analysis

PHB and carbohydrates were measured after each experimental phase. Samples were taken at the beginning of the 15 h light cycle and before C additions. 50 mL of mixed liquor was collected and centrifuged (4200 rpm, 10 min), frozen at −80 °C overnight in an ultra-freezer (Arctiko, Denmark) and finally freeze-dried for 24 h in a freeze dryer (−110 °C, 0.049 hPa) (Scanvac, Denmark).

PHB extraction and analysis were adapted from the methodology described by Lanham et al., (2013). Briefly, 1 mL of MeOH acidified with H_2SO_4 (20% v/v) and 1 mL of $CHCl_3$ containing benzoic acid as internal standard were added, between 2-3 mg of lyophilized biomass. Then tubes were then incubated in a dry-heat thermo-block (Selecta, Spain) during 5 h at 100 °C. After that, tubes were rapidly cooled on ice for 30 min. 0.5 mL of deionized water were added to each tube and they were vortexed for 1 min to separate the different solvents by density. $CHCl_3$ was removed with a Pasteur pipette and placed into a vial with molecular sieves to remove the water that could remain in the sample. PHB was determined by means of gas chromatography (GC) (7820A, Agilent Technologies,

USA). It was quantified measuring the PHB monomer hydroxybutyrate (HB) and hydroxyvalerate (HV), using the co-polymer of PHB-PHV as a standard for hydroxybutyrate (HB) and hydroxyvalerate (HV). A calibration curve with six points was prepared and processed in the same way as the samples.

Carbohydrate extraction was done following the methodology described by Lanham et al., (2012) and concentration was measured using the phenol-sulfuric acid method described by Dubois et al., (1956). Briefly, 2 mL of HCl (1N) were added to 2 mg of freeze-dried biomass, and then the tubes were incubated in a dry-heat thermo-block (Selecta, Spain) at 100 °C for 2h. Samples were left to cool down to room temperature (approximately 15 min) and then 0.5 mL of 5%$_{w/v}$ phenol solution and 2.5 mL of H_2SO_4 were added to the tubes. They were vortexed, then 10 min after placed in a bath at 35 °C for 15 min. Eventually the absorbance at 492 nm was measured by a spectrophotometer (Spectronic Genesys 8, Spectronic instrument, UK). Cyanobacteria synthetize carbohydrates as glycogen (Markou et al., 2012). However, the analytical methodology used, not only measured glycogen but also all the polysaccharides formed by glucose monomers, such as polysaccharides of the cell wall and the excreted exopolysaccharides.

3. Results and discussion

3.1. Isolation and purification

Isolation of cyanobacterial strains from photobioreactors treating wastewater was attempted using different culturing techniques and conditions. Colonies were formed and enhanced only under 1 klx illuminance. Different nutrient concentrations were tested using medium BG-11 at full strength or diluted 1:2.5 and 1:5. The highest concentration of nutrients present in full strength BG-11 medium (247 mg N-NO_3^-·L^{-1} and 5.6 mg P-PO_4^{3-}·L^{-1}) enhanced the cyanobacteria colony development over green algae. It is worth

noting that disaggregation of biomass flocs was necessary to achieve cyanobacteria isolation. In this case, the homogenization of samples using a Polytron™ was more effective than sonication of biomass in an ultrasonic bath. A low velocity and a short homogenization period (1min) were applied, so that cells were not damaged (Falcioni et al., 2006). This methodology has been effectively used in previous studies, such as, Ziglio et al., (2002), who used a homogenizer similar to the one used in this study to disaggregate sludge flocs. Four different species could be recovered as cyanobacterial monocultures. Further experiments were carried out only with the two unicellular species (*Synechocyistis* sp and *Synechococcus* sp.) due to their faster growth and easier handling.

Considering the likely presence of heterotrophic bacteria, a molecular characterization and identification of the two cyanobacterial cultures was achieved by 16S rRNA gene clone library analysis. A total of 48 clones from each library were analysed and grouped into OTUs at 99% of sequence similarity (more information can be found in the electronic annex). The S*ynechococcus* sp.associated community presented 6 different clones, including members of *Alphaproteobacteria* (relative abundance of 23.4%, OTUs 2 and 6), *Betaproteobacteria* (8.5%, OTU 3) and *Bacteroidetes* (48.9%, OTUs 4 and 5). Interestingly, a single clone classified within the Class *Cyanobacteria* (OTU 1, 19.1%). According to RDP this clone was classified (100% probability) within the genus GpIIa, and presented the highest sequence identity (99.7%, ncbi-BLAST) with *Synechococcus* sp. PCC8966. Regarding the S*ynechocystis* sp. associated community, 8 different clones were detected. Five of those clones were classified within the *Alphaproteobacteria* (31.3%, OTUs 13 – 17), one within the *Betaproteobacteria* (39.6%, OTU 11) and another within the phylum *Bacteroidetes* (18.8%, OTU 12). A single clone was classified within the Class *Cyanobacteria* (OTU 10, 10.4%), likely allocated within the genus GpIX (76% probability). Homology comparison against NCBI database showed the highest sequence

identity (99.9%) with *Synechocystis* sp. YACCYB507, and close relationship with the well-known *Synechocystis* sp. strain PCC6803 (99.8%). Despite the presence of typical accompanying heterotrophic microbiota, these results confirmed the successful enrichment of two monocultures of individual cyanobacterial strains and the preliminary morphological identification achieved by microscopic observation.

3.2.PHB and glycogen accumulation experiments

3.2.1. Biomass growth rate

The 2.5L-PBRs with monocultures started with a biomass concentration of around 100 mg VSS·L^{-1} (Figure 2 A). Both cultures grew until reaching values between 750 mg VSS ·L^{-1} and 1000 mg VSS ·L^{-1} by the end of the growth phase. Molecular analysis based on 16S rRNA clone libraries using *Synechocystis* sp. reactor biomass, revealed a clear shift in the microbial community composition. The relative abundance of the heterotrophic community was reduced, from 89.6% in the inoculum to 40% at the end of the process. Interestingly, this reduction in abundance of the accompanying microbiota was associated with their replacement by other members of *Bacteroidetes* (15.2%, OTU 18) and *Alphaproteobacteria* (23.9%, OTUs 19 and 20), suggesting that the operation conditions favoured development of alternative heterotrophic communities. Concomitantly, a drastic increase in OTU 10, corresponding to *Synechocystis* sp. was observed (from 10.4% to 60.9%), becoming the predominant member of the community. The molecular analysis of the *Synechococcus* sp. reactor showed similar behaviour. The relative abundance of OTU 1, corresponding to *Synechococcus* sp., increased from 18.8% in the inoculum to 69.8% in the bioreactor biomass. As a consequence, the abundance of heterotrophic bacteria reduced from 81.2% to 30.2%, and this decrease was also associated with a succession in the major phylotypes present. In the reactor, OTUs 2 – 7 were not detected, and new members of *Alpha*- (OTU 10, 2%), *Beta*- (OTU 8, 23.3%) and *Gamma*- (OTU 9, 5%) subgroups of *Proteobacteria* gained relevance. These results indicate the

suitability of the feeding regime for cyanobacterial development in the reactor. Also, it could be hypothesized that the feast-famine feeding process, that favoured the intracellular accumulation of carbon storage polymers, probably resulted in a decreased exudation by cyanobacterial cells, which in turn resulted in a limited amount of dissolved organic carbon for the development of heterotrophic bacteria. It is worth noting that the microscopic observation of this bioreactor revealed the presence of protozoa from the beginning. In the initial stages, most protozoa were observed in the cyst form. However, in the course of the experiment some protozoa evolved to their active form, engulfing the small *Synechococcus* sp. cells. At this point, a clear aggregation of the biomass was observed, as a defensive strategy against protozoa to prevent further predation (Stal, 2017). Accordingly, despite the protozoa presence *Synechococcus* sp. were eventually able to grow and dominate the culture, as demonstrated by molecular analysis and microscopic observations. Protozoa contamination is practically unavoidable if these processes are scaled up to industrial scale, especially when small genus of cyanobacteria are used (Day et al., 2017; Troschl et al., 2018). In the case of the mixed PBR, nearly all nutrients had already been consumed in the HTH-PBR, which explains the higher initial biomass concentration (690 mg VSS·L^{-1}) and subsequent slower growth rate. Indeed, the biomass in the mixed reactor was reaching the stationary phase when it was inoculated in the lab scale reactor. Microscopic observations revealed that in the mixed culture, the population was mainly dominated by cyanobacteria classified as *Synechocystis* sp. and *Leptolyngbya* sp. The percentages of these two genera remained fairly constant for the entire experiment.

Growth rate, duplication time, nutrients uptake rate and yields observed in each culture are shown in Table 1. The observed growth rates are lower than those reported in previous papers (usually ranging from 0.3 to 1.3 d-1 for *Synechocystis* sp. (Calixto et al., 2018;

Gonçalves et al., 2016; Patel et al., 2018) and from 0.3 to 1.23 d-1 for *Synechococcus* sp. (Calixto et al., 2018; Lürling et al., 2013; Patel et al., 2018)). Gonçalves et al., (2016) studied the effect of light and temperature on growth rate, concluding that the optimal irradiance and temperature for *Synechocystis salina* was 180 µmol photon·m-2·s-1 PAR and 25 °C. In the present study, an average illumination of 2.1 klx (about 36 µmol photon·m-2 ·s-1 PAR) and a temperature of 30 ± 2 ºC were applied. This light limitation may explain the slow growth. However, light increase does not seem to affect the PHB accumulation. For instance, Dutt and Srivastava, (2018) reached a PHB concentration of 15.5% with a light intensity of 150 µmol photons·s^{-1}·m^{-2} while Kamravamanesh et al., (2019) achieved a 23% with a much lower light intensity (50 µmol photons·s^{-1}·m^{-2}).

Nutrients uptake

3.2.1.1. N and P uptake

The evolution of nutrients concentration is shown in Figure 2 B and C. *Synechococcus* sp. showed the highest N-NO_3^- uptake rate, followed by *Synechocystis* sp. A much slower rate was observed in the mixed PBR. The culture with the highest N-NO_3^- yield was the mixed culture, followed by *Synechococcus* sp. and *Synechocystis* sp. (Table 1). This means that mixed PBR was the most efficient assimilating N-NO_3^- to grow. A yellowish colour was detected in this reactor from day 31, indicating a clear chlorosis process. No colour changes were observed in the other reactors. This difference may be related to the previous growth phase, since the monocultures grew in a higher N-NO_3^- concentration than the mixed culture, leading to a higher accumulation of nitrogen in cells (see Figure 3. B 1-3). Indeed, the value of N/VSS (%) after the growth phase was higher than a 9% in the monocultures and below a 7% in the mixed culture.

P-PO_4^{3-} release was observed during the first 4 days of the experiment in all reactors (Figure 2 C), which can be directly related to pH. During the upscaling process, pH

reached values up to 10, which could have caused precipitation of dissolved P-PO_4^{3-} present in the inoculated biomass. When pH was maintained below 9, these precipitates were dissolved again, increasing the soluble P-PO_4^{3-} concentration. Figure 3. A1, A2 and A3 show that the % P/X in the biomass decreased from between 5-2% up to <1% in the reactors with *Synechocystis* sp. and *Synechococcus* sp. This decrease may be attributed to the dissolution of precipitated forms of P. As observed in Table 1, PBR with *Synechocystis* sp. had the highest specific P- PO_4^{3-} consumption rate (0.43 mg P· g VSS^{-1}· d^{-1}). Much higher specific consumption rates were observed by other authors in 16 L vertical flat plate photobioreactors (up to 460 mg N· g VSS^{-1} · d^{-1} for N uptake in *Synechocystis* sp. PCC6803 (Kim et al., 2011) which was again related to slow growth due to lack of light.

3.2.1.2.Carbon uptake

DIC evolution is shown in Figure 4. In the *Synechocystis* sp. reactor (Figure 4 A), during the growth phase, CO_2 injection for pH control led to a DIC increase. When N and P were almost depleted, pH control was changed from CO_2 injection to HCl additions in order to achieve DIC depletion and to start the feast-famine phase (C, N, P exhaustion). During the feast-famine phase, CO_2 additions were done for 3 h. In this PBR not all the DIC added was consumed during the 6 h of the feast cycle, but a low concentration of DIC was always present during feast-famine. Finally, in the feast phase, $NaHCO_3$ was added reaching 173 mg C·L^{-1}.

In *Synechococcus* sp. reactor (Figure 4. B), CO_2 was also used to control pH during the growth phase until nutrients were depleted. In that phase, DIC followed a similar trend to that of the *Synechocystis* sp. reactor. During feast-famine, an addition of $NaHCO_3$ was done every day at the beginning of the cycle. After 6 h, a really small amount of DIC was left (Figure 4. B.1), so DIC was completely exhausted during the famine cycle. During

the feast phase, an addition of $NaHCO_3$ was done to reach a high concentration of DIC (about 120 mg $C \cdot L^{-1}$). However, a quick drop in DIC was measured immediately after this addition due to precipitation. Indeed, a 33% of the DIC added was transformed into PIC at the end of the phase (Figure 3. C2).

In the mixed PBR, due to the initial high concentration of PIC, HCl was used to control the pH during the whole growth phase and, in consequence, a progressive decrease in TIC was observed (Figure 4. C). During the feast-famine phase $NaHCO_3$ was also injected at the beginning of each phase and consumed after the feast cycle (Figure 4. C.1). As shown in Figure 3. C3, a strong precipitation of the DIC added in the feast phase was also measured in this reactor (63% of the added DIC). Precipitation in both reactors were attributed to unexpected malfunctions in the pH control system, that caused a rise in pH and the subsequent formation of carbonate salts.

A small concentration of dissolved organic carbon (DOC) was observed in all reactors (Figure 3, C1, C2, C3). However, in all cases, the maximum DOC concentrations reached were below 65 mg $C \cdot L^{-1}$. This fact ensured that the PHB production was photoautotrophic, since heterotrophic bacteria need DOC loads higher than 300 mg $C \cdot L^{-1} \cdot d^{-1}$ to synthesize PHB (Arias et al., 2018b).

3.2.2. PHB and glycogen accumulation

PHB and glycogen bioaccumulation profiles are shown in Figure 5. At the beginning of the growth phase, glycogen content was about $10\%_{dcw}$ in both *Synechococcus* sp. and mixed reactors (not measured for *Synechocystis* sp.). In contrast, PHB was not detected in either of them. This confirms that glycogen, unlike PHB, can be accumulated even without N and P limitation (Dutt and Srivastava, 2018). During the feast-famine phase, PHB percentage increased gradually in all PBRs. However, glycogen percentages decreased in *Synechocystis* sp. reactor, suggesting that a conversion from

glycogen to PHB formation could be taking place. Differently, in *Synechococcus* sp. and mixed reactors, both glycogen and PHB concentrations increased, which could be related to the different levels of C. Eventually, addition of DIC in the feast phase led to a fast increase of both PHB and specially glycogen in *Synechocystis* sp. reactor (see Figure 5A). On the other hand, glycogen concentration in the mixed PBR decreased and slightly increased in *Synechococcus* sp. PHB concentration was stable during this period in *Synechococcus* sp. and mixed reactors. These observations suggest that when carbon is limited, glycogen is the first storage compound to be consumed, whereas PHB is a long-term storage compound.

All in all, these results suggest that the mechanisms for PHB and glycogen accumulation are related to DIC concentrations. Small amounts of DIC seem to be necessary for C conversion and high concentrations of DIC enhance mainly glycogen accumulation. when DIC is not available, glycogen is the first storage component to be consumed. The cause of these changes may be related with PHB and glycogen metabolic pathways (Figure 6). It should be noted that, under N limitation, proteins can not be synthetized, and the photosynthetically fixed carbon in the Calvin cycle is converted to lipids and carbohydrates, contributing to the enhancement of the NADH pool (Markou et al., 2012; Singh et al., 2017). Regarding phosphorus, it is known that the ADP-glucose phosphorylase, the enzyme that produces carbohydrates, is inhibited by inorganic phosphorus and activated by the 3-phosphoglycerate (Markou et al., 2012). On the other hand, inorganic phosphorus deficiency declines the ATP generation by degrading polyphosphate, creating an imbalance in the NADH/ATP ratio. This imbalance has been previously related to activation of PHB synthesis (Kamravamanesh et al., 2019; Singh et al., 2017). In this context, if we have a lack of DIC, glycogen (and PHB to a lesser extent), will be degraded to provide the needed C to synthetize the essential sugars for the cell to

continue alive. If there is a small amount of DIC, this C can be used in the Calvin cycle to synthetize essential sugars. Then, glycogen could be degraded to pyruvate in order to produce more ATP, which can no longer be produced by means of polyphosphate degradation (Kamravamanesh et al., 2019). As far as amino acid synthesis is inhibited, this pyruvate will be transformed and stored as PHB. Finally, if there is an excess of DIC, the cell will store it mainly in the form of glycogen, as it is a more bioavailable carbon storage component. Further study in DIC kinetics in cyanobacteria metabolism should be done in order to enhance the PHB accumulation route instead of the glycogen route.

In a recent study by Arias et al., (2018a), a clear decrease in PHB and carbohydrates was observed during the dark phase, when DIC was completely depleted, whereas a conversion from carbohydrates to PHB was found when a small DIC concentration was present. C interconversion has been observed in recent studies (Arias et al., 2018a, 2018b; Dutt and Srivastava, 2018; Kamravamanesh et al., 2019; Troschl et al., 2018). For instance, Kamravamanesh et al., (2019) observed that when polyphosphate (PolyP) was no longer available, glycogen conversion to PHB occurred. Troschl et al., (2018) described that PHB accumulation occurred in 3 consecutive phases: a growth phase, a subsequent N limitation and glycogen and PHB production from CO_2, and a final interconversion of glycogen into PHB. However, the effect of DIC concentrations on the C metabolic pathways, which has been put in evidence in the present study, was not considered.

The maximum PHB and glycogen percentages were 5% and 68.9%, respectively, and they were both reached in the *Synechocystis* sp. reactor. In general, glycogen concentrations achieved in this study are in accordance with previous observations (Arias et al., 2018b; Kamravamanesh et al., 2019). Our results in PHB accumulation are similar to those obtained in previous studies with mixed cultures, but lower than those observed

in pure cultures. Arias et al., (2018b), obtained a maximum of 6.5% of PHB in a mixed culture dominated by cyanobacteria, fed with BG-11 medium, and an incubation period of 8 days. Regarding pure cultures, Kamravamanesh et al., (2019), reached a 23%$_{dcw}$ with *Synechocystis* sp. PCC 6714 in 16 days incubation, and BG-11 as a culture medium. Dutt and Srivastava, (2018) achieved a 15.5%$_{dcw}$ in 18 days with *Synechocystis* sp. PCC 6803, and BG-11 medium, and Nishioka et al., (2001), obtained a 55%$_{dcw}$ in a thermophilic *Synechococcus* sp in 11 days, using also BG-11. The low concentrations obtained may be related to the long cultivation periods. Indeed, more than 20 days were needed in all reactors to deplete N, P and C. As pointed out by Carpine et al., (2015), by the time these nutrients are depleted, other essential nutrients for PHB production may also be consumed, decreasing PHB accumulation and increasing the lysis rate. In order to increase PHB accumulation and make it industrially and commercially advantageous, it is essential to optimize the initial nutrient load in one step cultivation, so that, all nutrients added are consumed by the end of the exponential growth phase. This means ensuring a perfect coupling between nutrients and light availability. Another good strategy could be to naturally select individuals with a higher PHB productivity. Finally, another important consideration is DIC. As observed in this study, low DIC promotes the conversion of glycogen to PHB. Therefore, maintaining a low concentration of DIC during the accumulation phase could be a good strategy to promote PHB accumulation.

4. Conclusions

Wastewater-borne *Synechocystis* sp. and *Synechococcus* sp. were used for the first time to investigate PHB and glycogen accumulation mechanisms in cyanobacteria. The maximum PHB and glycogen percentages were 5%$_{dcw}$ and 68.9%$_{dcw}$, respectively, and were reached by *Synechocystis* sp. Results show that glycogen and PHB accumulation mechanisms strongly depended on DIC availability; when an excess of DIC is present

mainly glycogen but also PHB are boosted. On the contrary, when small amounts of DIC were present conversion from glycogen to PHB was evident. Results obtained in this study gave insight for new strategies to increase the %PHB in cyanobacteria.

Supplementary data

E-supplementary data of this work can be found in online version of the paper

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Figure Captions

Table 1. Growth rate, duplication time, nutrients uptake rate and yields in each culture

Figure 1. Graphical representation of the 2.5 L-photobioreactors' experimental set up. Colors represent the concentration of the main nutrients in each of the three experimental phases. The lighter the color, the lower is the concentration of the compound. Brown color represents the inorganic carbon concentration, blue the NO_3^- and green the PO_4^{3-}. Purple arrows indicate that pH control was done by means of CO_2 addition during that period and blue arrows indicate that pH control was done by HCl addition.

Figure 2. Concentration changes in volatile suspended solids (A), N- NO_3^- (B) and P-PO_4^{3-} (C) in all 2.5L-photobioreactors

Figure 3. Changes in different forms of phosphor (A), nitrogen (B) and carbon (C) in [$mg \cdot L^{-1}$] and the percentages of particulate organic phosphor, nitrogen and carbon in the biomass. A1, B1, C1 correspond to *Synechocystis* sp., A2, B2, C2, *Synechococcus* sp. and A3, B3, C3 to mixed.

Figure 4. Changes in inorganic carbon concentration for *Synechocystis* sp. (A), *Synechococcus* sp. (B) and mixed culture (C) reactors. Note that in the mixed culture reactor, the total inorganic carbon (TIC) (dissolved (DIC) + particulate inorganic carbon

(PIC)) was represented instead of only the dissolved inorganic carbon, as done in A and B, since in this reactor the PIC contribution was more important than the DIC. Insets A.1, B.1 and C.1 show the DIC changes observed in a feast-famine cycle on days 34, 25 and 24 respectively.

Figure 5. Changes in PHB and glycogen percentages for *Synechocystis* sp. (A), *Synechococcus* sp. (B) and mixed culture reactor (C). PHB and Glycogen were not measured at the beginning of the growth phase in PBR-*Synechocystis.*

Figure 6. Highly **s**implified biosynthetic pathways for glycogen conversion to polyhydroxybutyrate (PHB) in cyanobacteria. The pathways are reproduced and simplified with some modifications from (Kamravamanesh et al., 2018). Reactions with a cross represent the inhibited reactions due to N and P limitation. Green arrows represent the synthesis of PHB through the glycogen oxidation route. Abbreviations: 3-PGA, 3-phosphoglyceric acid; ADP, Adenosine diphosphate; ATP, Adenosine triphosphate; NADPH/NADP+ Nicotinamide adenine dinucleotide phosphate, NADH/NAD+, Nicotinamide adenine dinucleotide. (For interpretation of colour in this figure legend, the reader is referred to the web version of this article.)

Tables and figures

Tables

Table 1. Growth rate, duplication time, nutrients uptake rate and yields in each culture

Studied Genus	μ_X (d^{-1})	DT (d)	$q_{N\text{-}NO3}$ (mg N· g VSS^{-1}· d^{-1})	$Y_{X/N\text{-}NO3}$	$q_{P\text{-}PO4}$ (mg P· g VSS^{-1}· d^{-1})	$Y_{X/P\text{-}PO4}$
Synechocystis Sp.	0.083	8.4	5.89	14.07	0.43	191.03
Synechococcus Sp.	0.087	7.99	8.69	15.07	0.27	492.94
mixed culture	0.021	32.6	0.85	25.10	0.05	433.02

Figures

Figure 1:

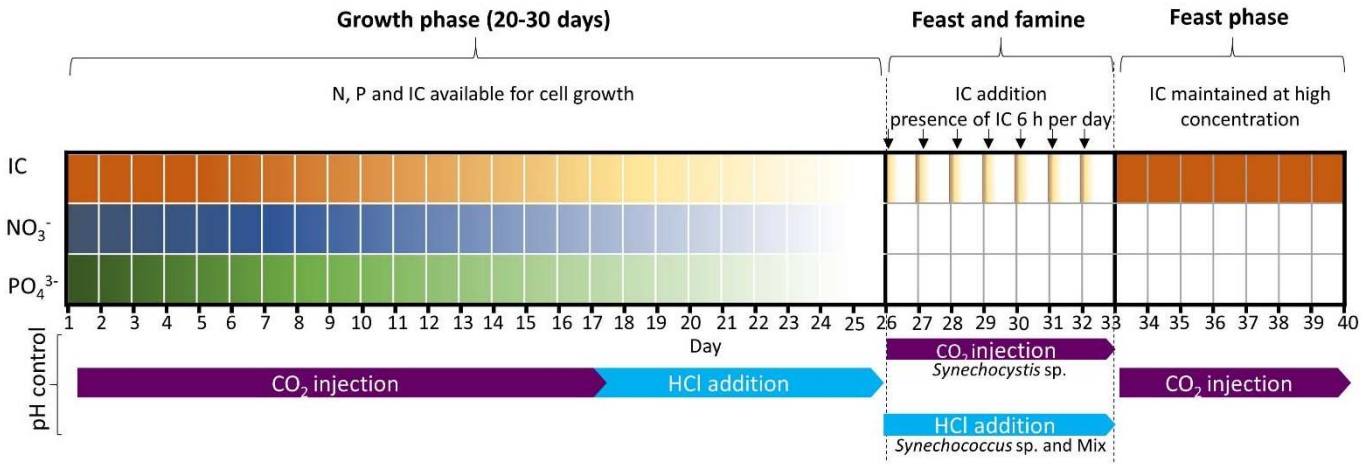


Figure 2:

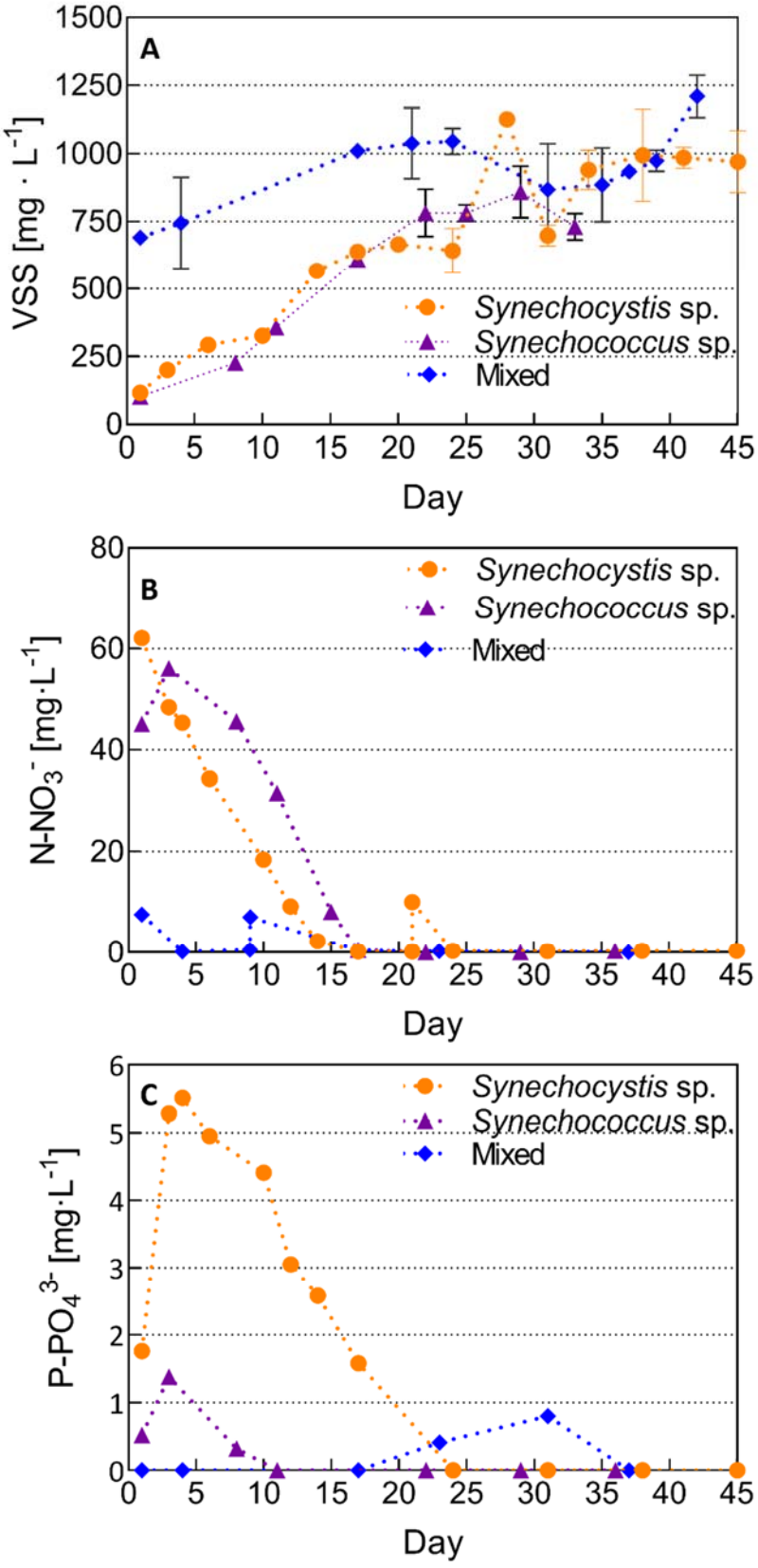

A
VSS [mg · L-1]
Day
Synechocystis sp.
Synechococcus sp.
Mixed
B
N-NO3- [mg·L-1]
C
P-PO43- [mg·L-1]

Figure 3.

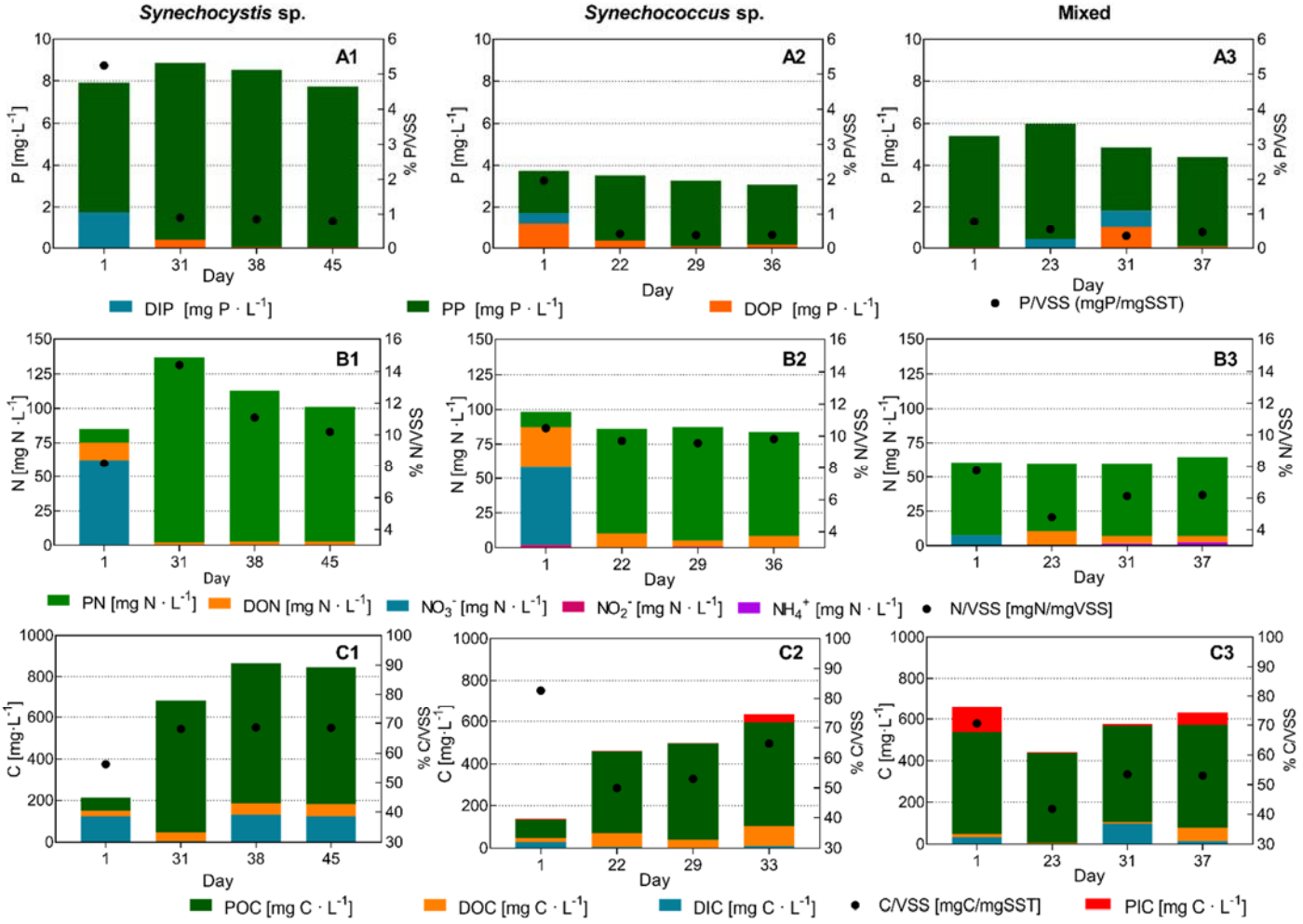

Synechocystis sp.
Synechococcus sp.
Mixed
A1
A2
A3
B1
B2
B3
C1
C2
C3
P [mg·L-1]
% P/VSS
N [mg N ·L-1]
% N/VSS
C [mg·L-1]
% C/VSS
Day
DIP [mg P · L-1]
PP [mg P · L-1]
DOP [mg P · L-1]
P/VSS (mgP/mgSST)
PN [mg N · L-1]
DON [mg N · L-1]
NO3- [mg N · L-1]
NO2- [mg N · L-1]
NH4+ [mg N · L-1]
N/VSS [mgN/mgVSS]
POC [mg C · L-1]
DOC [mg C · L-1]
DIC [mg C · L-1]
C/VSS [mgC/mgSST]
PIC [mg C · L-1]

Figure 4:

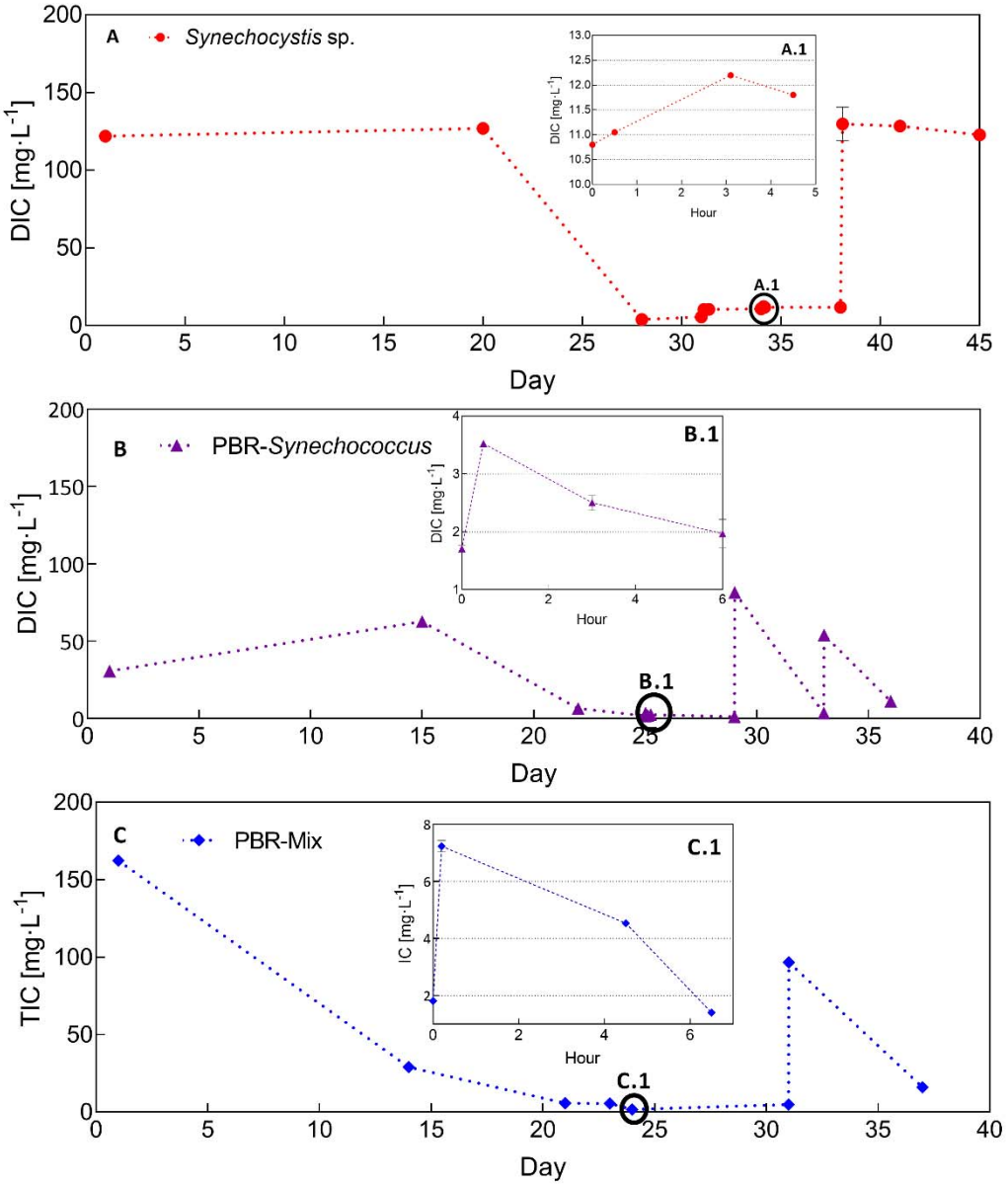

A
Synechocystis sp.
A.1
DIC [mg·L-1]
Hour
Day
B
PBR-Synechococcus
B.1
C
PBR-Mix
C.1
TIC [mg·L-1]
IC [mg·L-1]

Figure 5:

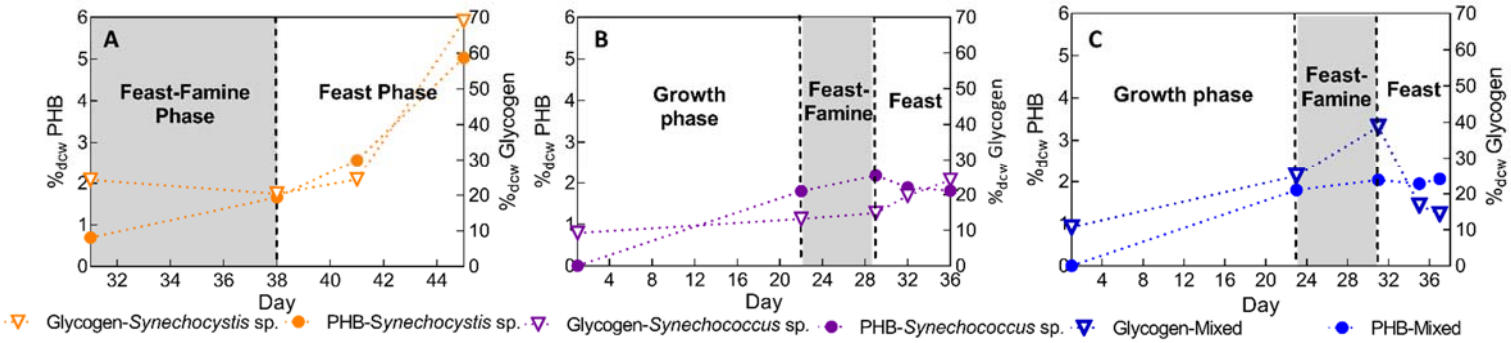
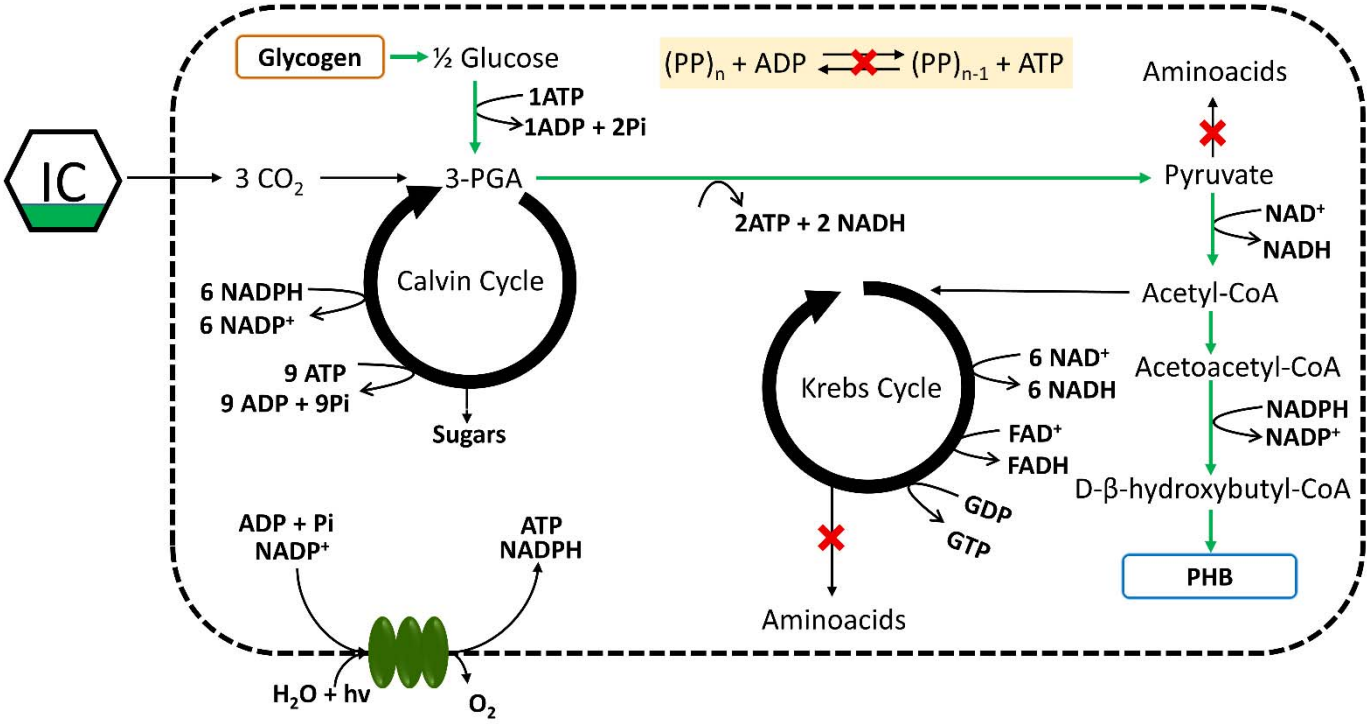

A
Feast-Famine Phase
Feast Phase
B
Growth phase
Feast-Famine
Feast
C
Growth phase
Feast-Famine
Feast
%dcw PHB
%dcw Glycogen
Day
Glycogen-Synechocystis sp.
PHB-Synechocystis sp.
Glycogen-Synechococcus sp.
PHB-Synechococcus sp.
Glycogen-Mixed
PHB-Mixed

Figure 6.


Glycogen
½ Glucose
1ATP
1ADP + 2Pi
(PP)n + ADP
(PP)n-1 + ATP
Aminoacids
IC
3 CO2
3-PGA
2ATP + 2 NADH
Pyruvate
NAD+
NADH
6 NADPH
6 NADP+
Calvin Cycle
Acetyl-CoA
9 ATP
9 ADP + 9Pi
Sugars
Krebs Cycle
6 NAD+
6 NADH
Acetoacetyl-CoA
FAD+
FADH
NADPH
NADP+
D-β-hydroxybutyl-CoA
GDP
GTP
ADP + Pi
NADP+
ATP
NADPH
PHB
Aminoacids
H2O + hv
O2